Pomegranate seed oil rich in conjugated linolenic acids reduces *in vitro* methane production

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(Received 13 October 2015; Accepted 10 July 2016; First published online 1 October 2016)

Abstract

The objective of this study was to determine the effect of pomegranate (*Punica granatum* L.) seed oil (PSO) on gas and methane (CH_4) production, ruminal fermentation and microbial populations under *in vitro* conditions. Three treatments consisting of a control diet containing 10 mg tallow (CON); the control diet with 5 mg PSO + 5 mg tallow (MPSO) and the control diet containing 10 mg PSO (HPSO) were compared. Ten mg of the experimental fat/oil samples were inserted into a gas-tight 100 mL plastic syringe containing 30 mL of an incubation inoculum and 250 mg of a basic substrate of a hay/concentrate (1/1, w/w) mixture. *In vitro* gas production was recorded over 0, 2, 4, 6, 8, 10, 12 and 24 h of incubation. After 24 hours, incubation was stopped, and methane production, pH, volatile fatty acids (VFAs) and microbial counts were measured in the inoculant. Gas production at 4, 6, 8, 10, 12 and 24 h incubation, metabolizable energy and *in vitro* organic matter disappearance increased linearly and quadratically as level of PSO increased. Furthermore, the 10 mg PSO (HPSO) decreased CH_4 production by 21.0% compared with the control (CON) group. There were no significant differences in total and individual VFA concentrations between different levels of PSO, except for butyric acid. After 24 h of incubation, methanogenesis decreased in the HPSO compared with the MPSO and CON treatments. In addition, total bacteria and protozoa counts increased with rising PSO levels, while population methanogenesis declined significantly. These results suggested that PSO could reduce methane emissions, which might be beneficial to nutrient utilization and growth in ruminants.

Keywords: Gas production, methane, *Punica granatum* L, ruminal fermentation, rumen microbes
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Introduction

Methane produced during anaerobic fermentation in the rumen represents feed energy loss and contributes to the greenhouse effect in the environment. Therefore, reducing methane emission has been an important objective for ensuring the sustainability of ruminant production. Methane is produced normally during fermentation of feed by methanogenic archaea. The removal of protozoa could also reduce methane production because some populations of methanogens remain attached to protozoa (Hess *et al*., 2003).

Plant oil extracts that are rich in unsaturated fatty acids could alter rumen fermentation. The composition of fatty acids and the degree of saturation in plant oils are important factors that could affect the rumen fermentation process. Saturated fatty acids are less toxic than unsaturated ones, which may inhibit fermentation in the rumen more intensively (Jenkins, 1993). A reduction in methane production with the addition of unsaturated fatty acids has shown that these fatty acids could serve as electron acceptors during biohydrogenation in the rumen (Hegarty, 1999). Because long-chain and medium-chain fatty acids are less fermentable than short-chain fatty acids, they may reduce the percentage of CH_4 produced in ruminants (Johnson & Johnson, 1995). Fats and oils impair CH_4 production. A number of mechanisms may be responsible for this effect, possibly as a direct consequence of medium-chain fatty acids or because long-chain polyunsaturated fatty acids are toxic to protozoa, and because fats and oils cause general impairment of fermentation (Beauchemin *et al*., 2008; Hook *et al*., 2010). A reduction in hydrogen accumulation by the

URL: http://www.sasas.co.za
ISSN 0375-1589 (print), ISSN 2221-4062 (online)
Publisher: South African Society for Animal Science
http://dx.doi.org/10.4314/sajas.v46i3.13

addition of unsaturated fat seems to be a promising procedure to reduce rumen CH_4 production. However, because the effects of fats are dependent on their composition, when incubated with rumen fluid, only oleic (C18:1n-9), linoleic (C18:2n-6) and α-linolenic (C18:3n-3) acids reduced CH_4 production (Wu *et al*., 2013; 2015). Mechanisms by which lipid supplementation lowers CH_4 production have been proposed, including reduced fibre digestion, diminished dry matter intake, methanogenesis inhibition, inhibition of protozoa, and reduction of hydrogen that is used for fatty acid biohydrogenation.

Pomegranate (*Punica granatum* L.) is one of the oldest fruits and is from the punicaceae family. Around 55% - 60% of pomegranate weight is edible, of which 75% - 85% is juice and 15% - 25% seed (Seeram *et al*., 2006). Seeds of all varieties of pomegranate are rich sources of oil. Pomegranate seeds contain 6.6% - 24% oil (Khodami *et al*., 2014). The oil consists of palmitic acid (C16:0) and stearic acid (C18:0), both saturated fatty acids, oleic acid (C18:1), a monounsaturated fatty acid, linoleic acid (C18:2), and an isomer of linolenic acid or conjugated linolenic acid (C18:3), called punicic acid (9-cis,11-trans,13-cis or trichosanic acid), both polyunsaturated fatty acids (Khoddami & Roberts, 2015). Punicic acid (31% - 86%) is the dominant fatty acid in pomegranate oil, followed by linoleic acid (0.7% - 24.4%) and oleic acid (0.4% - 17.4%) (Khodami *et al*., 2014; Khoddami & Roberts, 2015). A study of four cultivars of Iranian pomegranate seed oil (PSO) showed that 7.7% - 8.1% of the fatty acids are saturated and 91.8% - 92.1% unsaturated (Dadashi *et al*., 2013). Thus, the researchers hypothesized that PSO, which is rich in punicic acid, would alter the methanogenesis count without detrimental effects on rumen fermentation. The literature review showed that research has not been conducted on the effects of PSO on the parameters of rumen fermentation. Thus, the aim of this study was to determine the impact of PSO on *in vitro* gas production, rumen fermentation and microbial accounts in goats.

Materials and Methods

Pomegranate seed oil was extracted in a laboratory from dried pomegranate seeds using hexane as a solvent. The fatty acid profile of PSO was identified with gas liquid chromatography. The results are presented in Table 1.

Table 1 Fatty acid composition (%) of pomegranate seed oil

Fatty acid	Amount (%)
C14:0	0.42
C16:0 (palmitic acid)	4.85
C16:1 (palmitoleic acid)	0.14
C18:0 (stearic acid)	3.86
C18:1 n-9 (oleic acid)	10.40
C18:2n-6 (linoleic acid)	7.38
C18:3n-3 (α-linolenic acid)	0.22
CLNA	72.56
C20:0 (arachidic acid)	0.17
SFA	9.13
MUFA	10.54
PUFA	80.16
n-3PUFA	72.78
n-6PUFA	7.38
n-6/n-3	0.10

SFA: saturated fatty acids (sum of C14:0+C16:0+C18:0+C20:0);
MUFA: monounsaturated fatty acids (sum of C16:1+C18:1n-9);
PUFA: polyunsaturated fatty acids (sum of C18:2n-6+ C18:3n-3+ CLNA);
CLNA: conjugated linolenic acid.

Rumen fluid was collected from four rumen-fistulated (Bar-Diamond, Parma, ID, USA) Kacang crossbred male goats, weighing 35.41 ± 0.84 kg, which received a diet of 50% alfalfa hay and 50% goat

concentrate (W/W) twice daily at 08:00 and 17:00 for three months. The concentrate consisted of corn (25.4%), soybean meal (19%), palm kernel cake (35.9%), rice bran (11.7%), palm kernel oil (5%), ammonium chloride (1%) and a vitamin and mineral mixture (1%). The rumen fluid from the goats was mixed on a volume basis, and filtered through four layers of cheesecloth. The incubation inoculum was prepared by diluting the rumen fluid with a buffer solution according to Fievez *et al.* (2005) in a 1 : 4 (v/v) ratio. Mixed inoculums were stirred in a water bath at 39 ºC with purging CO_2 until use (10 - 15 min later). The three treatments were CON (control diet, without PSO, adjusted with 10 mg tallow), MPSO (control diet with 5 mg PSO, adjusted with 5 mg tallow) and HPSO (control diet with 10 mg PSO). As substrate *ca.* 250 mg (1 mm ground) of a mixture of alfalfa hay and concentrate (1/1, w/w) was placed in gas-tight 100 mL plastic syringes, and 30 mL of the incubation inoculum was added. Then samples containing 10 mg tallow, 5 mg PSO + 5 mg tallow, and 10 mg PSO were injected into the syringes of the CON, MPSO and HPSO treatments, respectively. All air was expelled from the syringes, after which their tips were closed. The syringes were placed in an incubator at 39 ºC for 24 h. Volumes of the gas produced were determined after 0, 2, 4, 6, 8, 10, 12 and 24 h incubation, and syringes were shaken carefully to ensure complete mixing of the contents. The experiment was repeated as three runs on three separate days with six replicates to estimate gas production.

Metabolizable energy (ME, MJ/kg DM) and *in vitro* organic matter disappearance (IVOMD) were estimated according to Menke *et al*. (1979) as:

OMD (g/kg OM) = 148.8 + 8.89 GP + 4.5 CP + 0. 651 XA

ME (MJ kg/DM) = 2.20 + 0.136 GP + 0.057 CP,

where OMD is OM disappearance
ME is metabolizable energy
CP is crude protein in g/100 g DM
XA is ash in g/100 g DM
GP is the net gas production (mL) per 200 mg sample.

Table 2 Chemical composition of substrate used for *in vitro* incubation

Chemical composition	g/kg
Dry matter	911.0
Organic matter	928.5
Crude protein	185.0
Ether extract	53.7
Neutral detergent fibre	375.2
Acid detergent fibre	225.0

After 24 h of incubation, methane production was measured by injecting 1 mL headspace gas from each of the syringes into a gas-liquid chromatograph (Agilent 5890 Series gas chromatograph, Wilmington, Delaware, USA) equipped with an FID detector. Separation was achieved with an HP-Plot Q column (30 m × 0.53 mm × 40 m) (Agilent Technologies, Wilmington, Delaware, USA) with nitrogen (99.9% purity) (Domnick-Hunter generator, Domnick-Hunter, Leicester, UK) as the carrier gas at a flow rate of 3.5 mL/min. An isothermal oven temperature of 50 ºC was adopted in the separation. Calibration was completed using standard methane prepared by Scotty Specialty Gases (Supelco, Bellefonte, Philadelphia, USA). All the procedures were repeated three times. The pH of the contents of the syringes was determined with a pH electrode (Mettler-Toledo Ltd., Salford, England).

The volatile fatty acid (VFA) content of the incubated rumen fluid was determined with gas chromatography. The incubated rumen fluid was fixed with 25% metaphosphoric acid, centrifuged at 3000 rpm, and the supernatant collected. The supernatant (0.5 mL) was added to an equal volume of 20 mM methyl n-valeric acid (Sigma Chemical Co., St. Louis, Missouri, USA). Separation was done on a Quadrex 007 Series (Quadrex Corporation, New Haven, CT 06525 USA) bonded phase fused silica capillary column (15 m, 0.32 mm ID, 0.25 µm film thickness) in an Agilent 7890A gas-liquid chromatograph (Agilent

Technologies, Palo Alto, Calif, USA) equipped with a flame ionization detector. The injector/detector temperature was programmed at 220/230 ºC, respectively. The column temperature was set in the range of 70 - 150 ºC with temperature programming at the rate of 7 ºC/min increments to facilitate optimal separation. Peaks were identified by comparison with authentic standards of acetic, propionic, butyric, isobutyric, valeric, isovaleric and 4-methyl-n-valeric acids (Sigma, St. Louis, Mo., USA), as described by Ebrahimi *et al*. (2014).

After 24 h of incubation, 1 mL rumen fluid from each of the syringes was used for DNA extraction. Total DNA was extracted from 0.4 mL homogenized rumen fluid using the QIAamp DNA Stool Mini Kit (Qiagen Inc., Valencia, Calif.) according to the manufacturer's protocol. The extracted DNA was stored at −20 ºC until used. Plasmid DNA from each group of microorganisms used for the preparation of a standard curve and the purity and concentration of plasmid DNA in each sample were measured with a spectrophotometer and the number of copies of a template DNA per mL of elution buffer were calculated according to the following formula, which is available online at the University of Rhode Island Genomics and Sequencing Centre (www.uri.edu/research/gsc/resources/cndna.html).

$$\text{Number of copies} = \frac{\text{Amount of DNA } (\mu\frac{\text{g}}{\text{mL}}) \times 6.022 \times 10^{23}}{\text{Length (bp)} \times 10^{9} \times 650}$$

Standard curves were constructed using CT values, which were obtained from a serial dilution of plasmid DNA from each bacterial group.

Primers that were used to quantify the populations of groups of microorganisms, were adapted from Ebrahimi (2012):

Table 3 Microorganism sequences of primers

Microorganism	Sequence 5' – 3'
Total bacteria F[1]	CGGCAACGAGCGCAACCC
Total bacteria R[2]	CCATTGTAGCACGTGTGTAGCC
Methanobacteriales F (MBT857F)	CGWAGGGAAGCTGTTAAGT
Methanobacteriales R (MBT1196R)	TACCGTCGTCCACTCCTT
Total protozoa R	GCTTTCGWTGGTAGTGTATT
Total protozoa F	CTTGCCCTCYAATCGTWCT

[1] forward, [2] reverse.

Rumen fermentation parameters and rumen microbes were analysed by one-way ANOVA, using the MIXED procedure of the SAS software package, version 9.1 (SAS, 2003). The following model was used: Yij = l + Di + Sj + eij, where Yij represents an observation; l is the overall mean; Di is the effect of the treatment (i = 1,2,3); Sj is the replicate, and eij is the residual error. Means were separated using the PDIFF option of the least squares means statement of the MIXED procedure. Linear and quadratic contrasts were used to determine the effects of increasing amounts of PSO on the response variables. Data were checked for normality using the UNIVARIATE procedure of SAS software (SAS, 2003). The results are presented as means ± standard error of the mean.

Results

The results of *in vitro* gas production are presented in Table 4. Methane production is illustrated graphically in Figure 1. The results showed that the gas produced (mL/250 mg DM) after 2 h incubation was not affected by PSO (P >0.05). However, gas production at 4, 6, 8, 10, 12 and 24 h incubation increased linearly and quadratically with cumulative PSO (P <0.05). *In vitro* organic matter disappearance and metabolizable energy were affected by PSO. Metabolizable energy (MJ/kg DM) and IVOMD (g/kg OM) increased linearly and quadratically (P <0.05) with accumulative PSO. Methane production of HPSO was higher (P <0.05) than CON, but not significantly different from the MPSO (P >0.05).

Table 4 Effect of pomegranate seed oil on *in vitro* gas production (mL/250 mg DM) after 24 hours' incubation

Incubation time(h)	Treatments			SEM	*P*-values	
	CON	MPSO	HPSO		Linear	Quadratic
2	4.33	3.33	4.00	0.484	0.792	0.448
4	6.17	4.33	9.50	0.642	0.004	0.001
6	9.33	6.17	13.67	0.855	0.001	<0.001
8	12.50	9.17	18.50	1.03	<0.001	<0.001
10	16.17	13.00	23.67	1.20	<0.001	<0.001
12	18.67	16.0	26.67	1.27	<0.001	<0.001
24	28.17	25.0	38.67	1.78	0.002	0.004
ME	6.32	5.97	7.46	0.194	0.002	0.004
IVOMD	436.41	413.88	511.09	12.68	0.002	0.004

CON: control (10 mg tallow); MPSO: medium pomegranate seed oil (5 mg tallow, 5 mg PSO);
HPSO: high pomegranate seed oil (10 mg PSO) after 24 h *in vitro* incubation;
ME: metabolizable energy (MJ/kg DM); IVOMD: *in vitro* organic matter disappearance (g/kg OM).

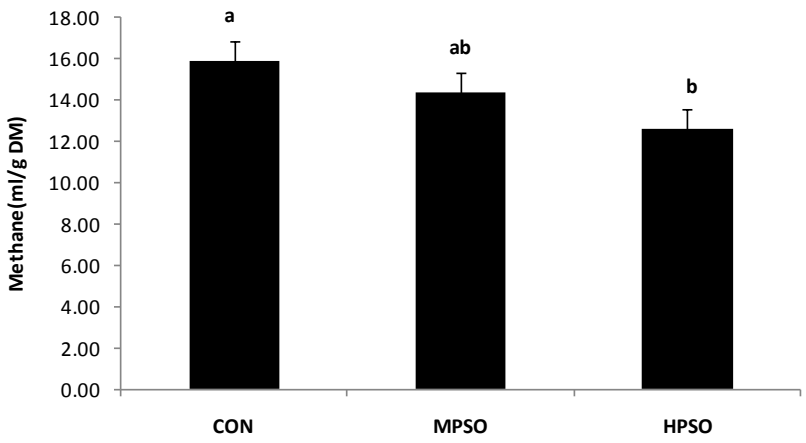

Figure 1 Methane production in experimental treatments.
CON: control (10 mg tallow); MPSO: medium pomegranate seed oil (5 mg tallow, 5 mg PSO), HPSO: high pomegranate seed oil (10 mg PSO) after 24 h *in vitro* incubation. Data presented as means with pooled standard error of the mean. Mean values sharing no common superscript are significantly different at $P < 0.05$.

The results of VFA and pH are presented in Table 5. Total VFA was not affected by PSO levels ($P > 0.05$). Acetate, propionate, valerate, iso-valerate, iso-butyrate and acetate to propionate ratios were not affected ($P > 0.05$) by PSO treatments, while butyrate concentration decreased linearly and quadratically ($P < 0.05$) with increasing PSO. The pH (7.18, 7.16 and 7.26) for the CON, MPSO, and HPSO diets, respectively, was not affected ($P > 0.05$) by oil supplements after 24 h incubation.

The results of the bacteria and protozoa count in the rumen are presented in Table 6. However, total bacteria numbers increased ($P < 0.001$) linearly, whereas methanogenesis decreased ($P < 0.001$) linearly with raised PSO. However, they did not show a quadratic effect by increasing the POS in the diet. The total number of protozoa showed linear and quadratic increases ($P < 0.001$) with augmenting the dietary levels of PSO.

Table 5 Effect of pomegranate seed oil on total and individual volatile fatty acid (VFA) production (mol/100 mol) and pH after 24 h incubation

	Treatments			SEM	*P*-values	
	CON	MPSO	HPSO		Linear	Quadratic
Total VFA (mmol/g DM)	9.68	9.72	10.01	0.273	0.950	0.617
Individual VFA (mol/100 mol)						
Acetate	59.36	59.72	61.21	0.548	0.792	0.170
Propionate	24.99	25.50	25.60	0.442	0.662	0.719
Isobutyrate	1.91	1.73	1.73	0.106	0.516	0.727
Butyrate	10.19	9.12	8.48	0.232	0.019	0.005
Isovalerate	1.68	2.09	1.15	0.266	0.543	0.212
Valerate	1.86	1.84	1.82	0.120	0.958	0.894
Acetate : propionate	2.39	2.37	2.40	0.054	0.926	0.904
pH	7.18	7.16	7.26	0.037	0.417	0.490

CON: control (10 mg tallow), MPSO: medium pomegranate seed oil (PSO) (5 mg tallow, 5 mg PSO);
HPSO: high pomegranate seed oil (10 mg PSO) after 24 h *in vitro* incubation.

Table 6 Effects of pomegranate seed oil on rumen bacteria and protozoa concentration (log_{10} copy no/L) of rumen fluid

Log_{10} copy no/L of rumen fluid	Treatments			SEM	*P*-values	
	CON	MPSO	HPSO		Linear	Quadratic
Total bacteria	11.96	12.23	12.69	0.057	0.0001	0.122
Total methanogenesis	7.90	7.76	7.43	0.051	0.0001	0.268
Total protozoa	6.25	6.33	7.47	0.101	0.0001	0.0001

CON: control (10 mg tallow), MPSO: medium pomegranate seed oil (PSO) (5 mg tallow, 5 mg PSO);
HPSO: high pomegranate seed oil (10 mg PSO) after 24 h *in vitro* incubation.

Discussion

In the current study, gas production at the various incubation times increased with higher levels of PSO, except at 2 h. The rise in asymptotic gas production indicated better nutrient availability for rumen microorganisms (Mahala & Fadel Elseed, 2007). However, the current results indicated that *in vitro* gas production of mixed 50% forage and 50% concentrate substrate increased with PSO, which is rich in punicic acid. In previous studies, Lee *et al*. (2001) showed the effects of long chain fatty acids on *in vitro* gas production by rumen anaerobic fungus and confirmed that *in vitro* gas production of filter paper was significantly increased by the addition of stearic, oleic and linoleic acids, but significantly reduced by the addition of linolenic acid. However, Wu *et al*. (2013) found that *in vitro* gas production of *Leymus chinensis* meal at 48 h using mixed rumen fluids of goats was decreased by the addition of oleic and linoleic acids. Similarly, *in vitro* gas production of mixed fine-ground corn and soybean meal at 4 h using mixed rumen fluids of steers decreased when adding 4% flaxseed oil (Quinn & Loe, 2008). In contrast, in another study Patra & Yu (2012) reported that coconut oil increased total gas production linearly. The variation in gas production between the results of the current study and previous studies might be due to different substrates, dosages and types of supplemented long-chain fatty acids and rumen microbes. In this study, methane production decreased quadratically with PSO supplementation. This may be because the high degree of unsaturation of PSO made it toxic to methanogens (Prins *et al*., 1972) and resulted in a strong decrease of a methanogenesis population. PSO is highly unsaturated, which could explain the mitigation of methane emission with PSO. Current researches have shown that methane production could be reduced with the

addition of oils rich in unsaturated fatty acids, such as soybean oil, sunflower seed oil, coconut oil and fish oil, which significantly decrease methane production owing to their biohydrogenation (Patra & Yu, 2012) and their toxic effects on methanogen bacteria (Benchar *et al*., 2014). A study by Lunsin *et al*. (2012) demonstrated that rice bran oil, which consists of high levels of oleic and linoleic acids, reduced methane production in lactating dairy cows. Patra & Yu (2012) indicated a quadratic decrease in methane concentration with supplementation of fish and coconut oils after *in vitro* gas production incubation. Similarly, Wu *et al*. (2015) noted that methane production decreased in response to linoleic acid supplementation at 12, 24 and 48 h *in vitro*. All these studies are in line with the current study, which suggests that methane emissions could be reduced owing to the direct effect of PSO on methanogenesis.

The estimated ME concentration (MJ/kg DM) of treatments was calculated from the amount of gas produced at 24 hours' incubation with a supplementary analysis of crude protein. The estimated ME (MJ/kg DM) and IVOMD (g/kg DM) were higher in the HPSO treatment compared with MPSO and the control.

Volatile fatty acids are major sources of metabolizable energy in ruminants (Van Soest, 1982). Reduction in VFA production would be nutritionally unfavourable for the animal. However, total VFA concentration (9.68, 9.72 and 10.01 mmol/g DM) for the CON, MPSO and HPSO treatments, respectively, was not affected (P >0.05), which suggests that this oil did not affect diet volatile acids. In a similar study, Ishlak *et al*. (2014) reported that supplementation of soybean oil, PSO and blackberry oil in a continuous culture system had no influence on total VFA. In another study, it was reported that fish and coconut oils had no impact on total VFA concentration (Patra & Yu, 2012). Butyrate decreased with PSO supplementation, confirming the results of Beauchemin *et al*. (2009), which indicated that butyrate level decreased with oilseed supplementation. The rise in molar proportions of butyrate may be the result of increased hydrogen in the rumen, which could be utilized by methanogenic bacteria to produce CH_4 (Moss *et al*., 2000). Therefore, reduced molar proportions of butyrate would be in agreement with the linear reduction in CH_4 formation with supplementation of PSO.

Similarly, the rumen fluid-incubated pH observed in this study was not influenced by diet, and was ideal for optimal rumen digestion and fermentation (Ørskov 1994). This observation is consistent with the results of Vargas *et al*. (2011), who observed that the addition of various vegetable oils (olive, sunflower, or flaxseed oils) did not alter pH concentration *in vitro*. The absence of detrimental effects of PSO could be owing to the nature of the oil. It has been reported that oil enhancement has minimal effects on rumen fermentation, despite their unsaturated nature (Palmquist & Conrad, 1980; Palmquist, 1991; Jenkins, 1993). Also, the moderate forage to concentrate ratio (50 : 50) employed in this study could be responsible for the lack of detrimental effects of the test oil on fermentation and microbial accounts.

In the current study, PSO, which is rich in punicic acid, had a positive effect on microbial growth. In addition, it increased total bacteria and protozoa. Other researchers have shown that flaxseed oil, which is rich in linolenic acid, did not affect the number of protozoa in the rumen fluid of cows (Doreau *et al*., 2009; Benchar *et al*., 2014). Ueda *et al*. (2003) reported that 3% flaxseed oil supplementation to a concentrate-rich diet (65%) fed to dairy cows did not alter the number of total protozoa. However, when 3% flaxseed oil was added to a forage-rich diet (65%), the number of protozoa did not change (Ueda *et al*., 2003). Based on these results, it appears that oil supplementation and basal diet cause shifts in the protozoal population. The reported influence of oils on rumen protozoa varies among studies, depending on the concentration, type, and fatty acid composition of the oil, as well as the nutrient composition of the diet (Ueda *et al*., 2003; Benchar *et al*., 2014). Supplementation with PSO resulted in linear decreases of methanogenesis. This is supported by Kim *et al.* (2008), who found that lipids inhibited methanogenesis owing to the toxicity of long-chain fatty acids to methanogenic bacteria (Henderson, 1973).

Conclusions

Pomegranate seed oil increased total bacterial and protozoa populations and decreased methanogenesis, which would be reflected positively in the form of decreased methane. PSO supplementation did not have negative effects on *in vitro* fermentation, although fermentation shifted within the whole incubation period and increased gas production.

Acknowledgments

The authors would like to thank the Faculty of Veterinary Medicine, Universiti Putra Malaysia.

Authors' Contribution

EM, ME, GYM, RJ, MFJ and AK contributed to the idea, design, and execution of the study. EM and ME performed the fatty acid analysis, while MFJ and EM contributed for the microbial work. ME and EM were responsible for the statistical analysis. All authors contributed equally to the write-up of the final manuscript.

Conflict of Interest Declaration

The authors declare that there is no conflict of interests regarding the publication of this article.

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